EFFECTS OF THE DEEPWATER HORIZON OIL SPILL ON INDIGENOUS MICROBIAL COMMUNITIES IN PENSACOLA BEACH SANDS

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Biology in the
School of Sciences

Georgia Institute of Technology
May 2013

EFFECTS OF THE DEEPWATER HORIZON OIL SPILL ON INDIGENOUS MICROBIAL COMMUNITIES IN PENSACOLA BEACH SANDS

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ACKNOWLEDGEMENTS

I would like to acknowledge BP/The Gulf of Mexico Research Initiative and the National Science Foundation for supporting this research.

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SUMMARY

The destruction of the Deepwater Horizon (DH) oil rig discharged approximately 4.9 million barrels of light crude oil into marine environments from April 20, 2010 to July 15, 2010. A significant amount of oil washed ashore on beaches in the Gulf of Mexico and was subsequently buried underneath layers of sand. The overall goal of this project was to investigate the temporal effects of oil contamination from the DH spill on indigenous microbial communities in Pensacola Beach sands. Shifts in the community composition of bacteria and archaea were determined using high-throughput sequencing of 16S SSU rRNA genes, and gravimetric analysis was used to quantify oil degradation by known oil-degrading taxa enriched and isolated from oil contaminated beach sands in the Gulf of Mexico. Amplicon sequencing revealed significant decreases in microbial diversity as well as a shift in the microbial community to Gammaproteobacterial and Alphaproteobacterial lineages (~80% of the community) in oil contaminated sands. Many of the dominant operational taxonomic units (OTU) detected in abundance in oiled sands showed high sequence identity to known oil-degrading bacteria. Clean sands were dominated by different Gammaproteobacteria and members of the Thaumarchaeota. Archaea were more abundant in uncontaminated sands and are thought to be inhibited by oiling. A succession of microbial populations was observed from known aliphatic degraders to polycyclic aromatic hydrocarbon degraders as components of the oil were preferentially degraded. Isolates enriched from oil contaminated sands degraded significantly more oil than uninoculated control groups, illustrating that ecologically relevant bacteria abundant in oil contaminated communities are capable of oil

degradation. Thus, we conclude that blooms of oil-degrading taxa are associated with the removal of hydrocarbons from the environment.

CHAPTER 1

INTRODUCTION

After the Deepwater Horizon (DH) oil rig exploded and sank, approximately 4.9 million barrels of light crude oil was released into the deep ocean and dispersed throughout the Gulf of Mexico (OSAT 2010). Although much of the surface oil was captured or dispersed, oil contamination remains onshore and on the ocean floor. The total effects of the oil spill on the marine environment are still unknown, but are expected to cause devastating damage to the marine ecosystems. Crude oils are composed of naturally occurring hydrocarbon compounds that are located in subsurface reservoirs, many of which occur beneath the seafloor. These hydrocarbon compounds can be divided into four compound classes: saturated hydrocarbons, aromatic hydrocarbons, asphaltenes, and resins. While saturated hydrocarbon compounds are comprised of long chains of carbon and hydrogen atoms that are readily biodegraded, aromatic compounds are composed of aromatic rings that are typically biodegraded at a slower rate (Head et al. 2006).

It is estimated that Gulf oil reserves exceed 46 billion barrels of crude oil, just a small fraction of the world's 1,470 billion barrels of oil (Anderson and Boulanger 2009) (CIA 2011). Oil hydrocarbons have been seeping naturally into the Gulf from the underground reservoirs for millions of years, and over time certain bacteria have evolved enzymatic pathways that are capable of metabolizing these hydrocarbons (*Microbes & Oil Spills*). Hydrocarbon-degrading bacteria have the ability to break down hydrocarbons

into carbon dioxide and water and can use the energy stored in the hydrocarbon bonds as an energy source (Head et al. 2006).

The impacts of oil contamination from the DH spill on Florida's beaches have been studied by the Kostka lab for nearly two years. The main focus of research has been on understanding the environmental and ecological controls on hydrocarbon degradation *in situ* (Kostka et al. 2011). Understanding how the microbial community composition and microbial abundances have temporally changed in response to oil contamination in beach sands would be beneficial for managing future oil spills. Although there has been ample research conducted on hydrocarbon degraders under laboratory conditions, there is a lack of field research on the response of indigenous microbial communities to oil contamination *in situ* (Head et al. 2006). Initial research has focused on the immediate or acute impacts of the oil spill on deep plume microbial communities surrounding the sunken oil rig, which noted an increase in overall bacterial abundance and showed that bacterial communities were dominated by known hydrocarbon-degrading taxa in the Gammaproteobacteria (Hazen et al. 2010). In addition, studies found that genes required for degradation of alkenes, alkynes, and cycloalkanes (all involved with hydrocarbon degradation) were more heavily expressed within oil plumes in the Gulf of Mexico (Lu 2012). However, despite this progress, the chronic or long-term effects of the oil spill on the microbial communities, especially in benthic environments, are still not understood.

The Kostka lab collected sediment cores from the supratidal and intertidal areas of Pensacola Beach, FL every six weeks from June 2010 to September 2011 (Kostka et al. 2011). The lab previously performed microbial community analyses on samples collected from June 2010 to September 2010. The goal of this research was to extend these initial

studies to examine microbial community succession and changes in diversity in sands from Pensacola Beach over time. Initial results show that bacterial abundance has increased between 2 to 4 orders of magnitude in oiled areas, and that bacterial communities from oiled sands are dominated by *Alcanivorax* spp. (Kostka et al. 2011). Twenty-four bacterial strains were also enriched, isolated, and purified from oil contaminated sands. Another goal of this research was to quantify oil degradation by a number of these strains in hopes of better understanding the controls and mechanisms of oil degradation by a pure culture. These findings will improve our understanding of microbial community responses to oil contamination and will be used to improve our response efforts to large-scale oil spills.

Based on ecological disturbance theories, we hypothesize that there will be an initial decrease in microbial diversity followed by succession of the communities and a rebound in diversity (Huston 1979). We will expect to observe a succession of microbial populations as as specific components of the oil are differentially degraded (Jimenez 2011).

METHODS

Sample site and collection

Sand samples were collected from municipal Pensacola Beach approximately every 6 weeks from July 2010 to June 2010 from the supratidal and intertidal areas of the beach as described in the accompanying paper (Kostka et al. 2011). Seven samples from were analyzed in this study and a detailed list is provided below (Table 1).

Table 1. List of samples used in study

Sample Name	Date	Depth	Oil Contamination
July 2010	July 30, 2010	30-40 cm	Yes
October 2010a	October 20, 2010	40-45 cm	Yes
October 2010b	October 20, 2010	24-36 cm	Yes
June 2011a	June 14, 2011	30-40 cm	No
June 2011b	June 14, 2011	30-40 cm	No
June 2011c	June 14, 2011	50-65 cm	No
June 2011d	June 14, 2011	50-65 cm	No

Sequence analysis

Total genomic DNA was extracted using the MoBio Powersoil DNA extraction kit. DNA extracts were assessed for purity using spectroscopic methods and genomic DNA was sent to the Argonne National Lab for multiplexed Illumina sequencing 16S SSU rRNA genes. A total of 160,432 sequences were obtained, with an average of 22,900

sequences per sample. Sequences were processed, and initial community analysis was performed using the software package Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al. 2010). Within QIIME, sequences were first clustered at 80% similarity to the Greengenes database using the uclust algorithm as a method of quality control. Sequences less than 80% similar to any sequence in the Greengenes database were removed. Sequences that passed were clustered again at 97% to the database. Sequences failing to cluster in the previous step were clustered de novo at 97%. Sequences that comprised less than 0.0001% of the total number of sequences were removed from the library as well. Representative sequences for each cluster were selected and an OTU (operational taxonomic unit) table was constructed. OTUs were assigned taxonomy using RDPclassifier. Beta diversity was assessed using Bray Curtis (a phylogenetic distance metric) and distances ordinated using non-metric multidimensional scaling plots. Dominant OTUs driving Bray Curtis distances were identified and correlated with the amount of oil contamination, date of sample collection, and depth.

Quantification of oil degradation

Twenty-four pure cultures were previously isolated from oil-contaminated sands from the Gulf of Mexico. Three strains, *Alcanivorax dieselolei, Acinetobacter sp.,* and *Marinobacter hydrocarbonoclasticus,* were tested for growth and degradation of Macondo crude oil. Cultures were grown in triplicate on 0.5% crude oil in an artificial seawater medium in the dark at room temperature. Uninoculated media with 0.5% crude oil was used as a control. Cultures were shaken constantly at 90 rpm and were periodically shaken by hand for further aeration for 14 days. At regular intervals,

bacterial growth was quantified by spectrophotometry. Oil-degradation was quantified through gravimetric analysis. The initial mass of the oil was measured before inoculation (about 20 mg), and after 14 days the cultures were extracted with an equal amount of chloroform. The chloroform-extractable layer was transferred into pre-weighed flasks then placed under an air-stream at 60°C for 24 hours then at 25°C until the residual chloroform was evaporated. The mass of the remaining oil was measured and compared to the initial mass to measure oil degradation. The percentage of oil removed was calculated and was compared between the control and treatment flasks using a one-way ANOVA test.

RESULTS

Microbial communities show rebound in diversity within one year of the spill

In order to illustrate how the native microbial communities responded to and rebounded from the DH oil spill, the environmental sequences were analyzed in a number of ways. Rarefaction curves show a distinct difference in microbial diversity both over time and corresponding to oil contamination. In oiled samples collected within 5 months of the spill, the diversity, measured as species richness, is approximately 50% lower than in clean samples collected almost one year after oil came ashore on Pensacola Beach. Microbial diversity is slightly lower in October 2010 samples than in July 2010 samples.

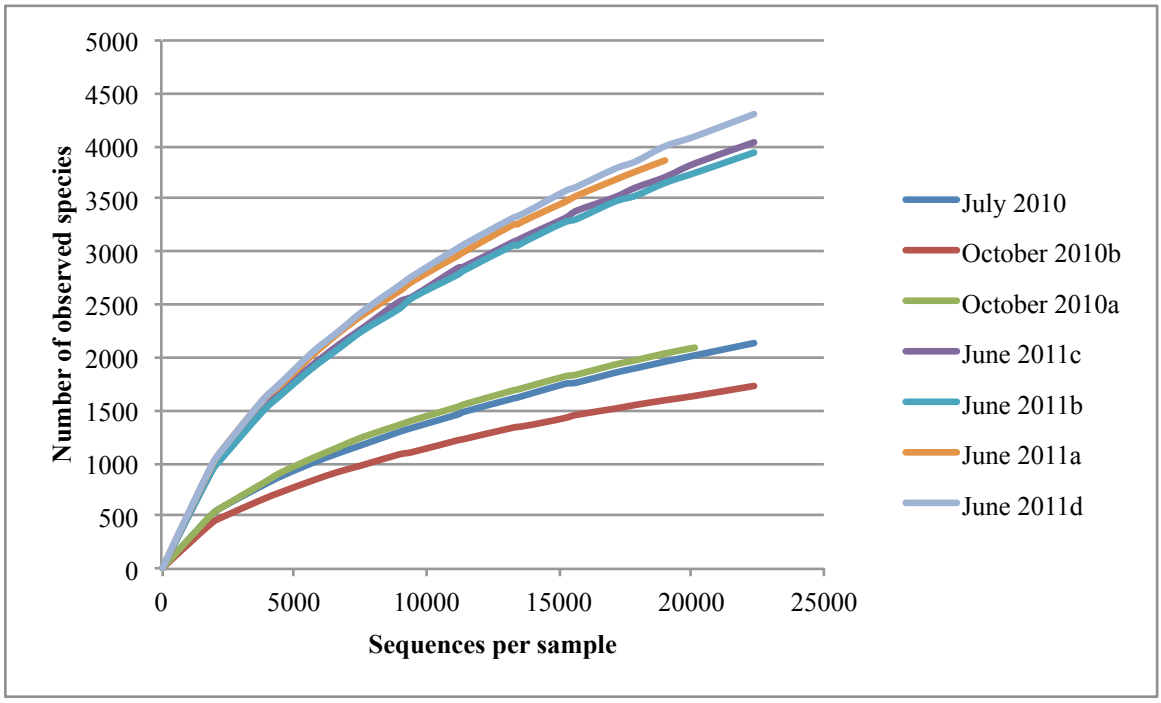

Figure 1. Rarefaction curves derived from SSU rRNA gene amplicons show a rebound in microbial diversity within one year of the DH oil spill. The microbial diversity within 5 months of the spill, when the sands are still contaminated with oil, is about 50% lower than microbial diversity is a year after the spill. At this point, the distinct oil layer seen in earlier months was no longer detectable.

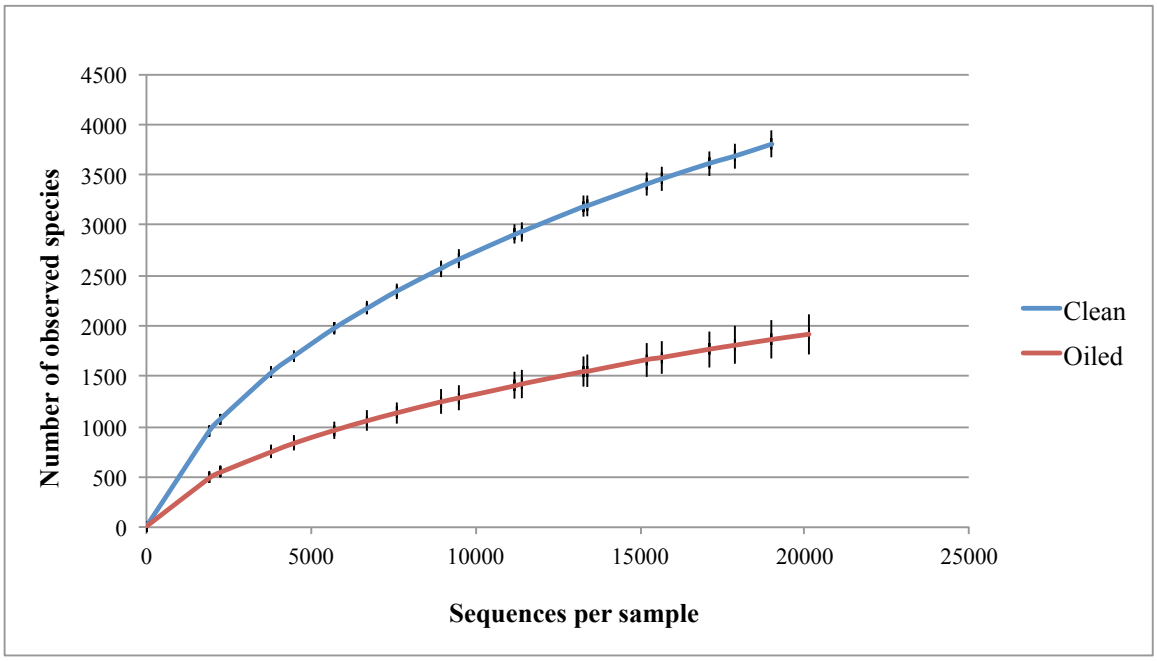

Figure 2. Rarefaction curves show microbial communities are less diverse in oil contaminated sands compared to clean sands. Diversity, measured as number of distinct species observed, is almost 2 times higher in clean samples than in oil-contaminated samples.

Community composition in oiled and clean beach sands

The community composition of oiled and clean sand samples also provides evidence for a rebound within one year after the spill. The 2010 samples are dominated by Gammaproteobacterial lineages, comprising on average approximately 50% of the total community. There was also an increase in the relative abundance of taxa known to degrade oil. In July 2010, members of the genus *Alcanivorax* made up 7% of the total community, members of the genus *Marinobacter* made up 3% of the community, and members of the genus *Oleibacter* made up 3% of the community. We see a succession in the communities of oil degraders between July and October 2010 and a shift in microbial communities between oiled and clean samples.

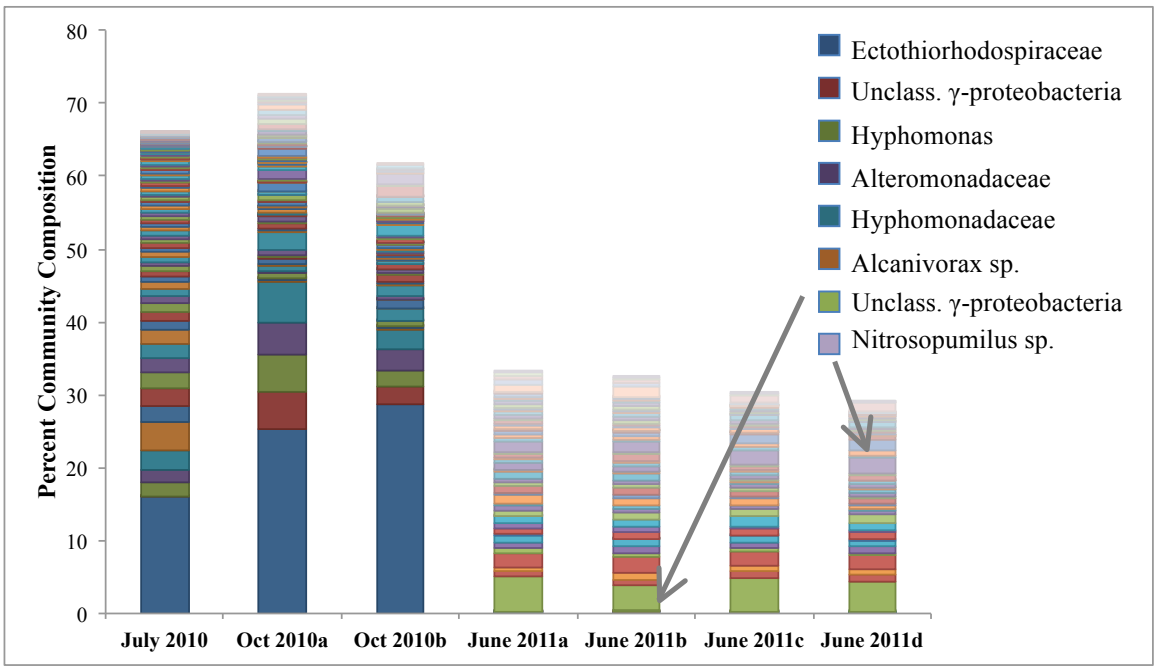

Figure 3. Community composition of oiled and unoiled beach sands based on abundant OTU. The top 30 OTUs were chosen from both the July and October 2010 samples, and the top 70 OTUs were chosen from the July 2011 samples. Only 125 of the total 9953 OTUs are represented above. The 2010 communities are dominated by Gammaproteobacterial taxa and are less diverse than communities from unoiled sands in 2011.

In the 2011 samples, the top 70 OTUs make up less than 35% of total community composition while the top 30-40 OTUs in the 2010 samples make up 60-70% of the total community composition, indicating a much more even and diverse community in 2011. Top OTUs in the 2011 samples are different Gammaproteobacterial lineages than are seen in 2010 samples as well as some Thaumarchaeotes.

Oiled communities are dominated by Gammaproteobacterial and Alphaproteobacterial lineages (~80% of the community). There is an order of magnitude difference between Alphaproteobacteria abundances in oiled sands compared to clean sands (~30% compared to ~3%). Many of these organisms are members of the families Rhodobacteraceae and Hyphomonadaceae.

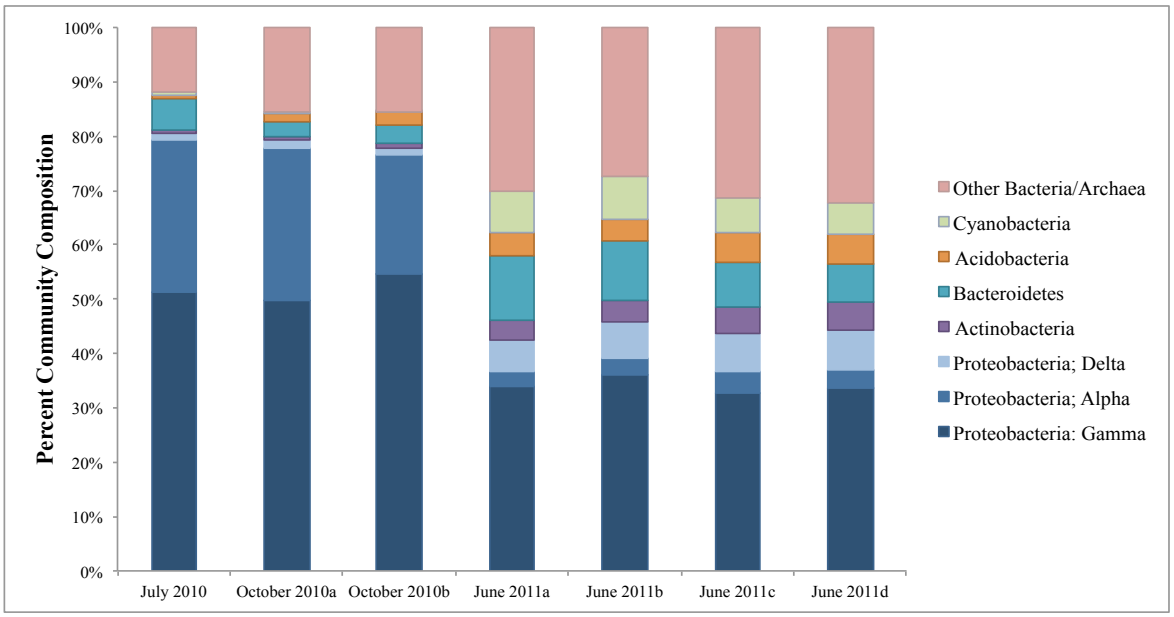

Figure 4. Community composition at the phylum level. Oiled communities are dominated by Gammaproteobacterial and Alphaproteobacterial taxa. There was a large increase of Alphaproteobacteria in oiled samples (~30% of the community).

Archaea are less abundant in oiled sands. In oil-contaminated communities, Archaea represent on average 0.1% of the total community, whereas in communities from unoiled samples, Archaea make up about 7% of the total community.

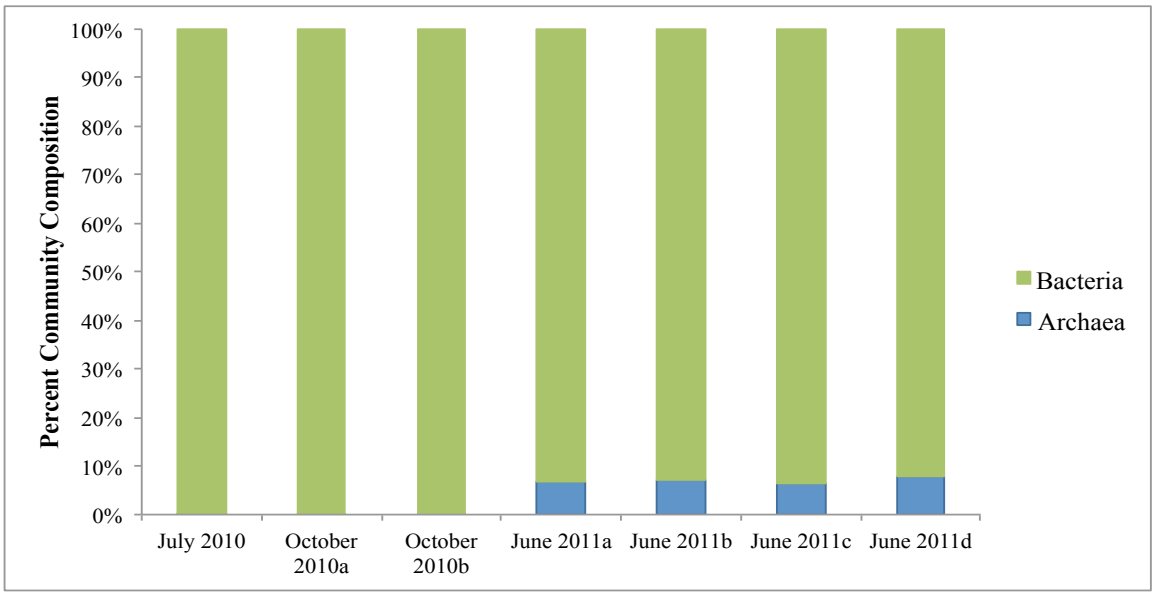

Figure 5. Community composition at the domain level. Archaeal communities are more abundant (7% of the community) in unoiled sands compared to oiled sands (0.01% of the community).

Beta-diversity was determined using Bray-Curtis similarity and was used to compare unoiled and oiled communities. A non-metric multidimensional scaling plot (NMDS) was used to illustrate the similarities between samples. More similar samples cluster together in space. The June 2011 samples are more closely related to each other than they are to October and July 2010 samples.

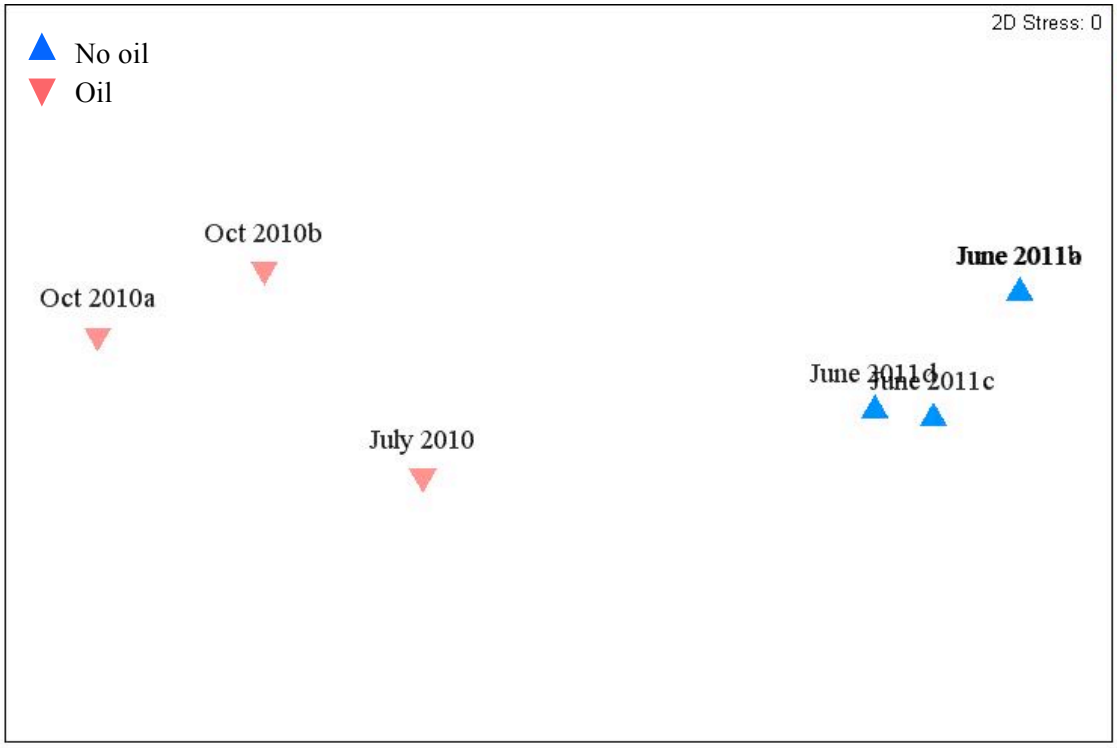

Figure 6. NMDS plot of Bray-Curtis similarities based on sampling date and presence of oil. Samples cluster first by the presence or absence of oil then by date. Oiled samples are more closely related to each other than to unoiled samples.

Quantification of oil degradation by bacteria isolated from Gulf of Mexico sands

Gravimetric analysis was used to quantify the amount of oil degradation carried out by bacteria that were isolated from contaminated Gulf of Mexico sands. Three strains, *Alcanivorax dieselolei, Acinetobacter sp.,* and *Marinobacter hydrocarbonoclasticus,* were first tested for growth on 0.5% Macondo crude oil using a spectrophotometer. *Acinetobacter* (COS2) grew the quickest and showed the highest amount of growth. The

spectrophotometer recorded little growth by *Marinobacter* (EN3); this is due to the fact that *Marinobacter* forms clumps as it grows. Therefore, spectrophotometry is not the best method for studying its growth. Cellular proteins would have been a better measure to quantify growth, or the use of a surfactant could be applied in the future to prevent clumping.

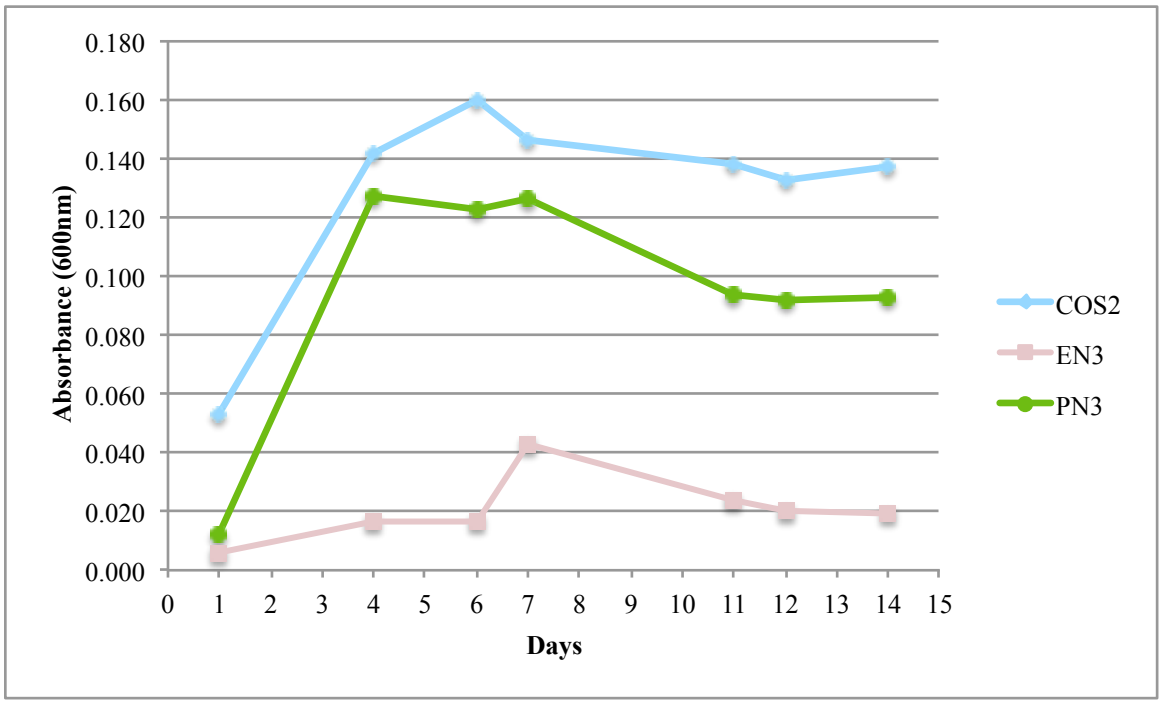

Figure 7. Growth of oil-degrading isolates quantified through spectrophotometry. *Acinetobacter* (COS2), *Marinobacter* (EN3), and *Alcanivorax* (PN3) were all able to grow on Macondo crude oil as the sole carbon source. *Acinetobacter* grew the quickest and had the highest growth out of the three isolates, n=1 for each isolate.

Within 2-4 days there is a noticeable transformation of the oil in treatments containing the bacteria, and by 14 days a significant proportion of the oil is removed. The pictures below qualitatively illustrate how the bacteria transform the oil as it is degraded. The oil sheen seen in the control (orange) below is quite different from the oil that has been acted upon by the bacteria. The oil is transformed into small, black droplets of what is thought to be asphaltenes and resins. Significantly more oil is removed in treatments

containing oil-degrading bacteria. In the control group, 33.74% (SD ± 3.77%) of the oil was removed through natural evaporation. With this accounted for, the bacterial treatments, on average, degraded an additional 20% of the oil. An average of 58.5% (SD ± 4.79%) of the oil was removed in flasks containing *Marinobacter*, 64.5% (SD ± 6.61%) with *Acinetobacter*, and 56.04% (SD ± 9.71%) with *Alcanivorax.*

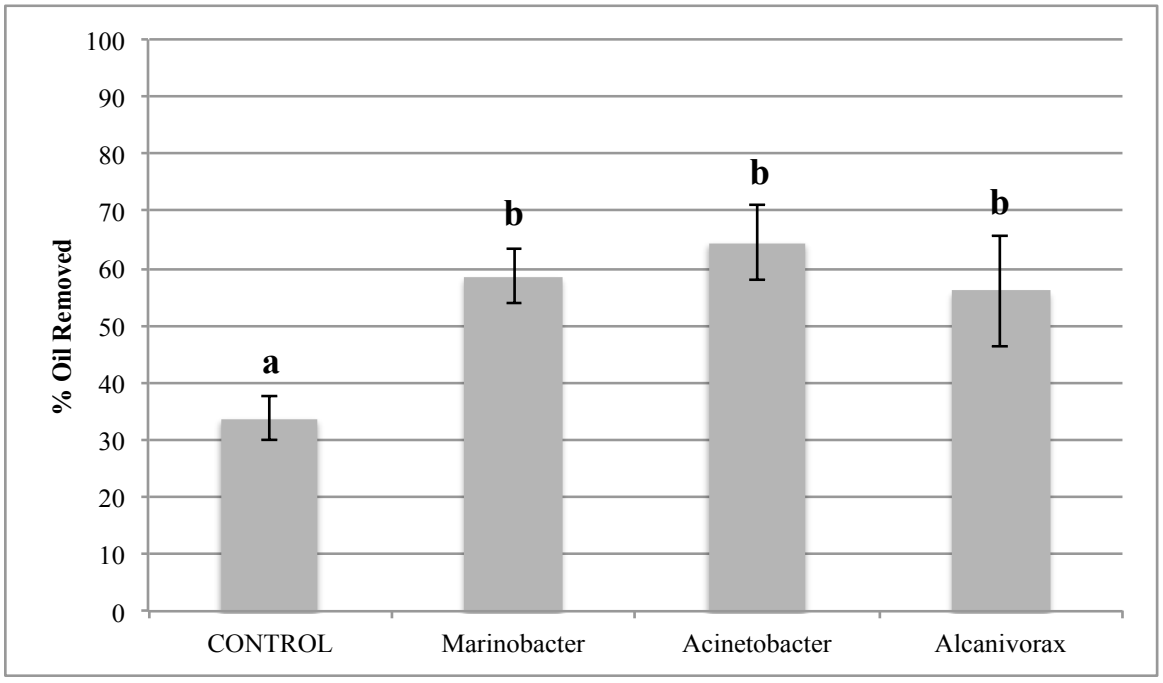

Figure 8. Significantly more oil is removed in groups inoculated with oil-degrading bacteria compared to control group. On average, 20% more oil was removed in treatments containing oil-degrading bacteria. A one-way ANOVA test was used to compare the total amount of oil removed in control and treatment groups. There was significantly more degradation in treatments containing bacteria ($p<0.05$). A student's t-test was used to compare between each group. There was significantly more degradation between all groups labeled A and B, but there were not significant differences in oil degradation within members of group B.

The difference in the initial and final masses of oil in the control and bacterial treatments was compared using a one-way ANOVA test. There was a significant difference in oil degradation ($p<0.05$) between the control and bacterial treatments, which was confirmed using a student's t-test between the control and each treatment. The t-test verified that there wasn't a significant difference in oil degradation between the bacterial

treatments, but there was a significant difference in oil degradation between the control and each treatment group. A table listing the p-values is provided below (Table 2).

Table 2. P-values from student's t-test between treatment and control groups

	Control	***Marinobacter***	***Acinetobacter***
Control	-	-	-
Marinobacter	0.033	-	-
Acinetobacter	0.004	0.398	-
Alcanivorax	0.046	0.797	0.317

Figure 9. Qualitative assessment of oil degradation in treatment and control flasks. The pictures were taken after 14 days of incubation. Starting with the top left corner and moving clockwise: *Marinobacter, Alcanivorax,* control, *Acinetobacter.* A significant proportion of the oil is transformed and removed in flasks with bacteria.

DISCUSSION

Hydrocarbon-degrading bacteria are ubiquitous in marine environments, but are usually only present at low detection levels. In response to oil contamination, however, these microbes have been shown to increase in relative abundance up to 70-90% of microbial communities (Röling et al. 2002). The removal of oil hydrocarbons from the environment is known to be heavily dependent on biodegradation by these microbial communities (Leahy and Colwell 1990). Few studies, however, focus on the *in situ* response of indigenous microbial communities to oil contamination. This study is unique in that it analyzes a time series, which allows for an understanding of how microbial communities in beach sands respond to and rebound from oiling. Ecologically relevant bacteria isolated from oiled beach sands were also tested to further understand the processes and pathways by which microbes remediate oil contamination.

In the accompanying study conducted in 2010, 454 pyrotag sequencing revealed that *Alcanivorax* was one of the initial responders to oil contamination and quickly dominated the communities. One species of *Alcanivorax* constituted up to 40% of the total community (Kostka et al. 2011). Within one month of oil coming ashore, total *Alcanivorax* species decreased to about 7% of the community and by October it comprised <1% of the community. Previous studies have observed a similar pattern with *Alcanivorax*; a quick initial colonization is followed by a collapse in bacterial numbers within a few weeks (Head et al. 2006). The decline in population size correlates with the removal of the majority of aliphatics (Kasai et al. 2001).

Members of the Proteobacteria, specifically Gammaproteobacteria and Alphaproteobacteria, are significantly enriched in oil contaminated beach sands. These two classes together comprise up to 80% of the total microbial community. This is consistent with previous studies showing that Alpha and Gammaproteobacteria predominate in oil-contaminated environments, mostly because the majority of known oil-degrading taxa belong to these lineages (Capello et al. 2006; Head et al. 2006). The sample from July 2010 is dominated by an Ectothiorhodospiraceae taxon (~16%) that was found to be 100% similar to a sediment clone sampled from an oil-polluted retention basin; therefore it is likely that the species is capable of oil degradation (Paissé et al. 2008). The community also consists of Gammaproteobacteria including *Alcanivorax, Oleibacter,* and *Marinobacter* as well as Alphaproteobacteria such as *Thalassospira.* These bacteria are known to specifically degrade aliphatic hydrocarbons (Jiminez et al. 2011; Teramoto et al. 2011; Yakimov et al. 1998).

Polycyclic aromatic hydrocarbons (PAHs), as mentioned earlier, are hydrocarbon compounds comprised of multiple aromatic rings and are more recalcitrant to biodegradation (*Microbes & Oil Spills*). These components are degraded more slowly than straight or branched chain aliphatics and are believed to be the more toxic compounds of crude oils. It is expected that as the more labile components of the crude oil are degraded, there would be a shift in the microbial communities to taxa that are capable of or more efficient at degrading the more recalcitrant compounds. Jimnez et al. conducted a three month long study with indigenous microbial communities collected from the Prestige oil spill to examine the changes in community composition in relation to the degradation of both aliphatics and PAHs. It was observed that changes in

community structure correlated with the chronological degradation of components in the oil; bacteria that most efficiently degraded the aliphatics were succeeded by bacteria that were capable of degrading the more recalcitrant PAHs (Jimenez et al. 2011). Many Alphaproteobacterial taxa detected in July and more prominently in October were known PAH degraders, including *Hyphomonas, Erythrobacter,* and *Parvibaculum* (Wang et al. 2008). *Hyphomonas jannaschiana*, a member of the Alphaproteobacteria, made up approximately 5% of total community composition in October samples. This strain has been linked to the degradation of PAHs (Wang et al. 2008). This supports our hypothesis that there is a succession of oil degrading taxa as the more labile components of the crude oil are degraded.

It has been shown that oil has an adverse effect on beach sand archaeal communities in laboratory microcosms, which is consistent with *in situ* observations. Archaea were virtually undetectable in microcosms amended with oil but were present in unoiled experiments (Röling et al. 2004). It was also observed through a bioassay that oil has toxic effects on the nitrite production of *Nitrosopumilus,* one of the most abundant archaeal taxa we observe in PB sands (Urakawa et al. 2010). It is likely that archaea are essentially undetectable in our oiled samples because the most abundant archaeal taxa are sensitive to oil contamination. It has been previously suggested that the presence of archaea could be utilized as an indicator of ecosystem recovery from oil contamination (Stephen et al. 1999).

Unpublished results from Markus Huettel's lab show a decrease in total petroleum hydrocarbon (TPH) compounds within one year of the oil spill. Concentrations of TPHs

return to background levels by January 2011. This removal of hydrocarbons is correlated with a clear succession of microbial communities and an overall increase in microbial diversity. The microbial communities in June 2011 are more even than the communities in July and October 2010. Results are not yet available for pre-oil spill communities at Pensacola Beach, but we are in the process of extracting and analyzing a series of these samples.

The initial decrease in microbial diversity following the DH oil spill was expected based on what is known about ecological theories of disturbance, which is described as an event that causes temporary and localized shifts in communities (Dornelas 2010). In ecological studies in which major disturbances have been observed, it has been noted that there is an immediate decrease in diversity followed by a succession and rebound in diversity (Huston 1979). This pattern is seen in all oil-contaminated environments (Head et al. 2006). In this study, there is an initial decrease in diversity in July followed by another slight decrease in diversity in October followed by a large rebound and increase in diversity in June 2011. Diversity is reduced because organisms capable of oil degradation strongly outcompete all other organisms incapable of utilizing the hydrocarbon compounds within the crude oil (Röling et al. 2004b). Once these compounds are depleted, conditions return to the pre-disturbance environment and the communities begin to rebound.

Many species are capable of oil degradation (Head et al. 2006). We were able to successfully show that ecologically relevant members of the microbial community enriched in oil-contaminated sediments are capable of degrading oil. Geochemical data

show a decrease in the concentration of total petroleum hydrocarbons within one year of the oil spill, which is concordant with an overall increase in total cell numbers as well as an increase in known oil-degrading taxa (Huettel, Unpublished data 2011; Kostka et al. 2011). We show that relevant members in this microbial bloom are capable of degrading components of the crude oil, so we can conclude that the majority of the oil contamination in the Pensacola Beach sands was remediated by indigenous members of the microbial community.

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